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#1 9/24/02

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT	:	ARNTZEN, et al
SERIAL NO	:	09/767,734
FILED	:	September 29, 2000
TITLE	:	VACCINES EXPRESSED IN PLANTS
Grp./A.U.	:	1638
Conf. No.	:	1914
Examiner	:	Collins, Cynthia E.
Docket No.	:	P00245USD

DECLARATION OF DR. JOHN HOWARD UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Madam:

I, John Howard, hereby declare and state:

1. I am the Chief Science Officer of ProdiGene, Inc., the assignee of the above-identified application. Previously, I was Director of Biotechnology Research for Pioneer Hi-Bred International, Inc. for seven years, and Director of the Protein Products Division for two years. For nine years prior to this, I initiated and directed a biotechnology research program for crop improvement with Stauffer Chemical Company.

2. I have a Ph.D. in Biochemistry from the University of California at Riverside. I have been involved in the science of the expression of proteins in plants for twenty years.

3. I have read the office action dated May 29, 2002 (USSN 09/767,734), and understand the Examiner is objecting to our claims regarding expression of viral antigens in plant cells, such as the viral antigen proteins from Hepatitis B and TGEV.

4. Experts have opined that expression of heterologous DNA in a plant is unpredictable. Fischhoff et al., WO 90/10076 pp. 1-13, states that expression of a heterologous gene in transformed plant cells might be so inefficient as to be without utility, due to incomplete transcription of the gene due to premature transcription termination; due to unexpected mRNA processing during transcription; inefficient or incorrect splicing or polyadenylation of mRNA;

instability of the cytoplasmic mRNA; inefficient translation of the cytoplasmic mRNA; or instability of the protein due to its susceptibility to plant proteinases.

5. While it may be hoped that a gene would fold properly in a plant environment, there is no reasonable certainty of this result. The plant intracellular environment differs from the intracellular environment where the protein is normally expressed. This would have particular consequence for protein folding. For example, a fish antigen protein expression was shown completely unstable when expressed in plants at normal temperatures. See Kenward et al., Plant Mol. Biol., 23:377-85 (1993). Further yet, the processing of the heterologous protein product in plants may produce an altered structure, or lead to miscompartmentalization, or be processed in low yields. This is particularly problematic with viruses, which rely on their host for processing. Since viruses are normally processed by the animal host cell, plant processing is not predictable based upon processing in the animal or in a bacteria. For examples of heterologous proteins modified by their in planta processing see Lee and Raikhel, Brazilian 7. Med. Biol. Res., 28:743-50 (1995).

6. TGEV is a glycosylated viral protein, and folding after protein production must match the active form of protein.

In addition, TGEV is a membrane bound protein. These types of proteins are more difficult to express than other types of proteins. Membrane proteins typically achieve only very low expression levels. As exemplified in Fryxell, K.H., et al., "Functional Expression of Soluble Forms of Human CD38 in *Escherichia coli* and *Pichia pastoris*", Protein Expression and Purification, 6:329-336(1995), the membrane protein Human CP38 demonstrates expression levels of only 0.05 g/L in *Pichia pastoris*. Weiss, H.M., et al., "Expression of Functional Mouse 5-HT SA Serotonin Receptor in the Methyltrophic Yeast *Pichia postoris*", Pharmacological Characterization and Localization, FEBS 377:451-456 (1995), observed expression at a level of 0.001 g/L for the membrane protein murine serotonin receptor in *Pichia pastoris*. This is in stark contrast to the regulatory protein tumor necrosis factor and the antigen Tetanus toxin (fragment C) which achieved expression levels of 10.0 g/L (Sreckrishna, K., et al., "High Level Expression, Purification, and Characterization of Recombinant Human Tumor Necrosis Factor Synthesized in the Methylotrophic Yeast *Pichia*

pastoris", Biochemistry, 28:4117-4125 (1989)) and 12.0 g/L (Clare, J.J., et al., "High-level Expression of Tetanus Toxin Fragment in *Pichia pastoris* Strains Containing Multiple Tandem Integrations of the Gene", Bio Technology, 9:455-460 (1991a)) respectively in *Pichia*. Such low expression levels would not lead one of skill in the art to expect that an immune response could be achieved with transgenic plants producing the antigen at these expression levels.

7. TGEV is a devastating disease to the swine industry and causes considerable economic losses. Welter, Joseph, "Comments on Vaccines for Transmissible Gastroenteritis", J.A.V.M.A., 160:4 pp 558-560 (1972). The data showing plant expression of TGEV S-Protein, which is disclosed in the present application, is an experiment which I have reviewed. In that experiment, expression of the TGEV S protein was confirmed. Despite suggestions for years to obtain TGEV expression, until the present application, there are no reports of it being expressed in plants. As described below, expression of the TGEV S protein was confirmed in further experiments using the protocols described in the application.

8. Hepatitis B is a serious health hazard in many countries and is considered one of the most widespread viral infections of humans. Tiollais, P. et al., Science, 213:406-411 (1981). Since it is a virus, it, like TGEV, cannot be predicted to be capable of expression in plants. Many of the factors recited above which make expression of TGEV unpredictable also are present with Hepatitis B. This too is a virus formed by a complex of proteins. To my knowledge, the results described in the present patent application are the first to demonstrate stable expression of the Hepatitis B antigen in plants.

9. I further had experiments conducted in which we were able to achieve high expression of viral antigens in plants. The procedures involved were those described in the specification of the 09/767,734 application. The details are outlined below.

One construct was created, and copies of the plasmid map is attached. The construct contained the gene coding for TGEV S-protein. Two-thirds of the gene sequence was optimized for plant expression, with the remaining one-third having the wild-type gene. As is evidenced from the plasmid map attached, the ubiquitin promoter was used to drive the gene, along with PinII (protease inhibitor gene from potato) as terminator. The selectable

marker is the maize optimized PAT gene, encoding resistance to bialophos, driven by the CaMV35S promoter. The genes were placed into the vector with necessary components for transformation with *Agrobacterium tumefaciens*. The *Agrobacterium* transformation methods of Ishida were used (Ishida et al., "High Efficiency Transformation of Maize (*Zea mays* L.) Mediated by *Agrobacterium tumefacient*", Nature Biotechnology, 14:745-750 (1996) and also described in U.S. Patent 5,591,616) with modifications as follows. The Hi II maize line was used which initiates Type II embryogenic callus in culture; selection was made on bialaphos medium; the EHA101 strain was used and re-inoculated after growing the bacteria overnight. The result is redifferentiation of the plant cells and regeneration into a plant.

Following the above procedure, the expression levels of TGEV were determined. In sum, the construct (TGEV S-protein) demonstrated expression levels of TGEV protein of up to 0.1% of total soluble protein in seed.

In the first construct, the expression levels were determined using the Western blot gels, copies of which are also attached. The Western blot is a procedure well known to those skilled in the art to analyze protein expression levels. *See e.g.*, Hood et al., "Commercial Production of Avidin from Transgenic Maize; Characterization of Transformants, Production, Processing, Extraction and Purification", Molecular Breeding, 3:291-306 (1997). From the amount of protein loaded on the gels it was determined, as summarized in table 1, that the expression level of the TGEV-S protein was up to 0.1% in seed.

10. I am unaware of any expression of animal viral antigens in plants earlier than that described in the present application and its parent applications, at any detectable level and to my knowledge, the expression levels outlined in the above paragraph are the highest levels of expression obtained for an animal viral antigen in plants.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on the information and belief are believed to be true; and further that these statements were made with the knowledge that the willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the

United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Dated this 11 day of September, 2002.

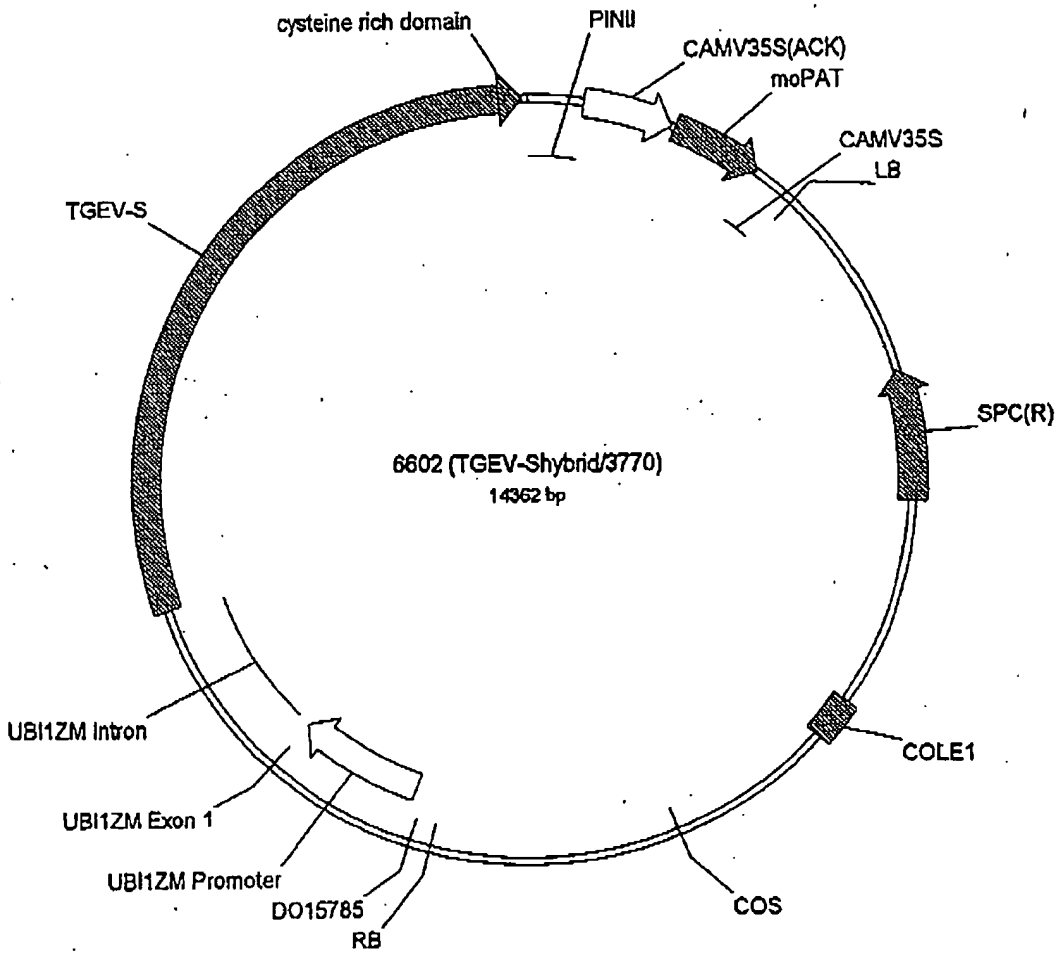
Dr. John A. Howard

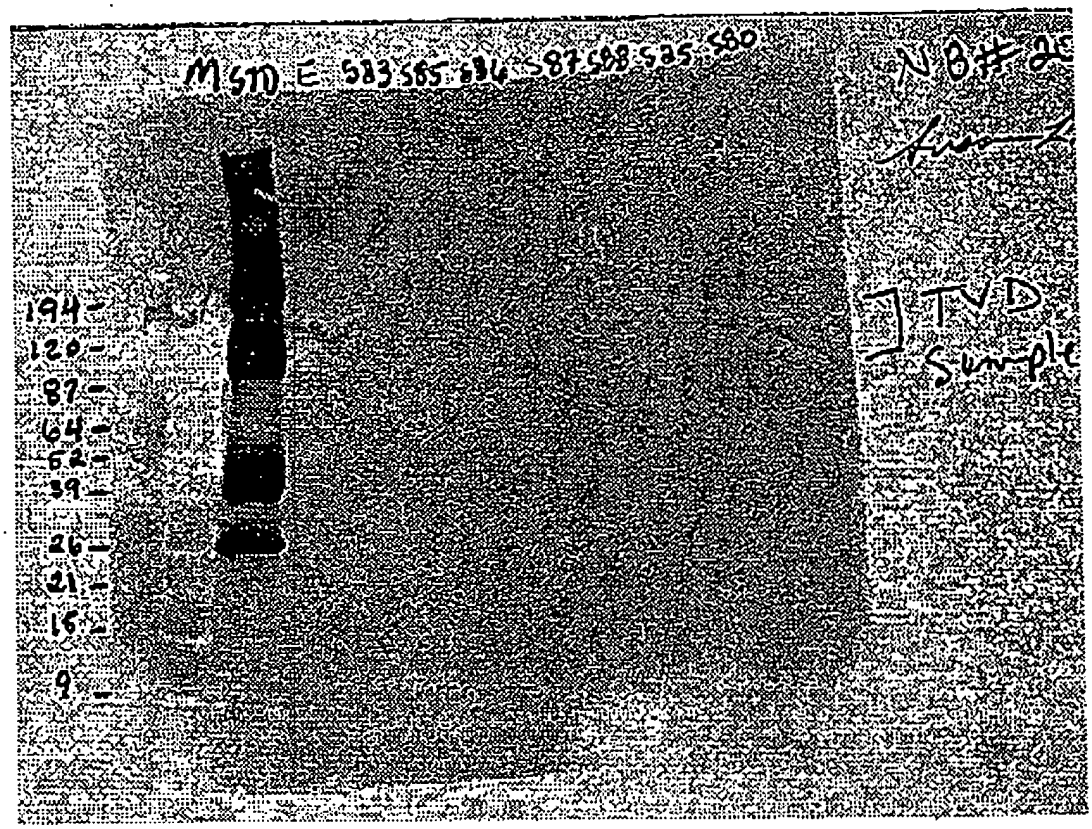
TABLE 1

TVD TGEV-S two thirds optimized

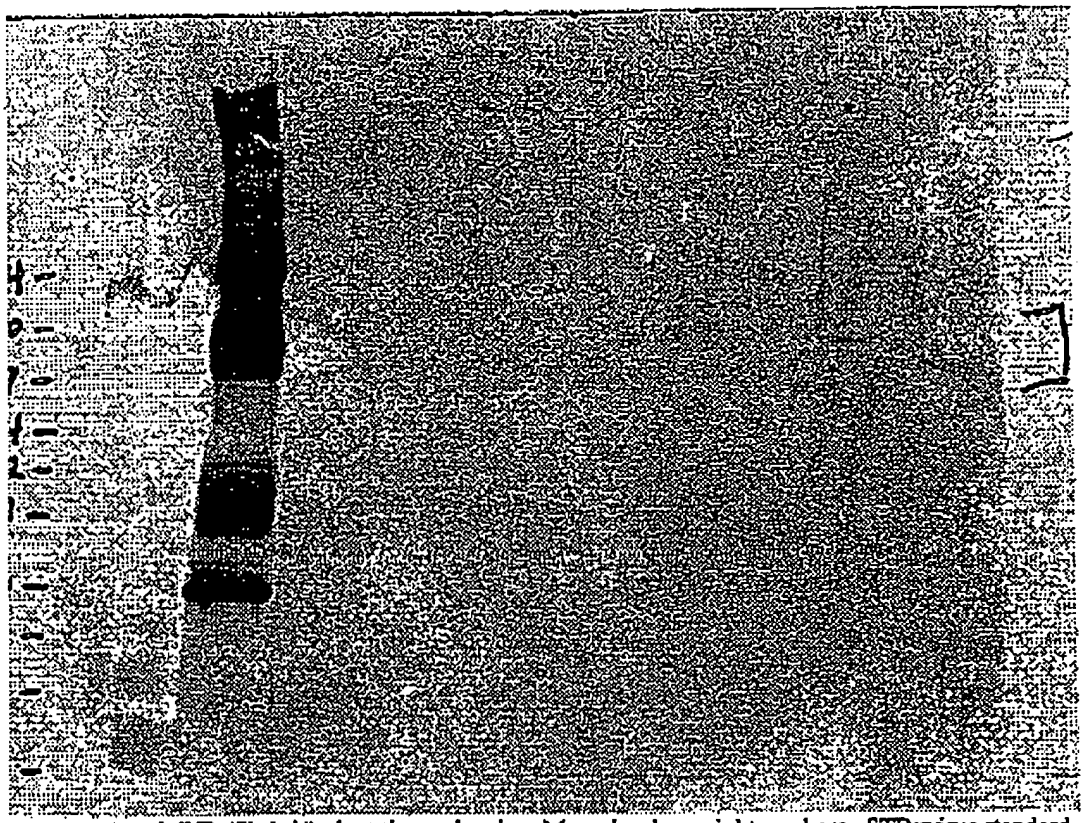
EVENT	LEAF pos./tot.	estimated range of expression	SEED pos./tot.	est. range of exp.
1	0 of 5		0 of 2	
2	0 of 13		0 of 4	
3	0 of 17		2 of 8	0.0300 -0.0400%
4	0 of 12		1 of 7	0.0050%
5	0 of 8		0 of 2	
6	8 of 12	0.0100%	no seed	
7	0 of 8		3 of 5	0.0100 - 0.1000%
8	0 of 18		2 of 10	0.0100 - 0.1000%
9	0 of 7		0 of 1	
10	0 of 3		1 of 1	0.0200%

12/11/98 05:18PM 6602 (TGEV-Shybrid/3770,

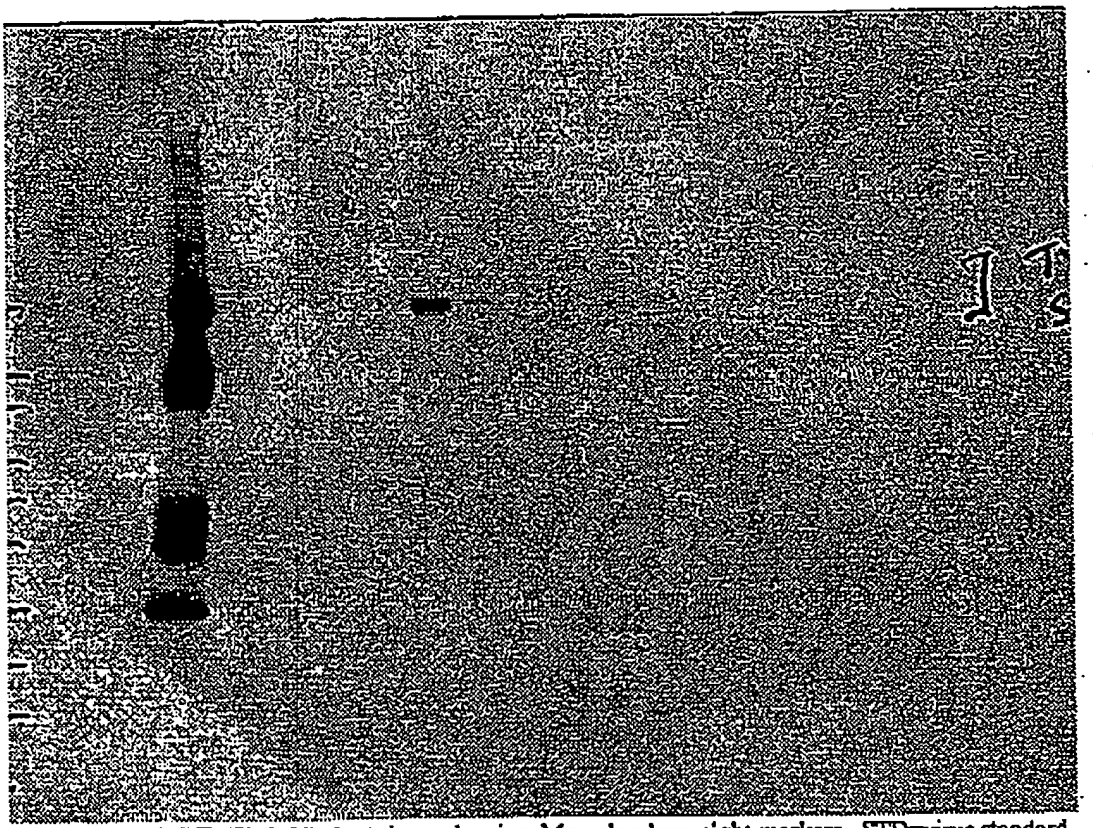




Western blot of TVD (Shybrid) plant tissue showing M=molecular weight markers, STD=virus standard, E=non-transformed plant tissue, the numbers represent individual plants.

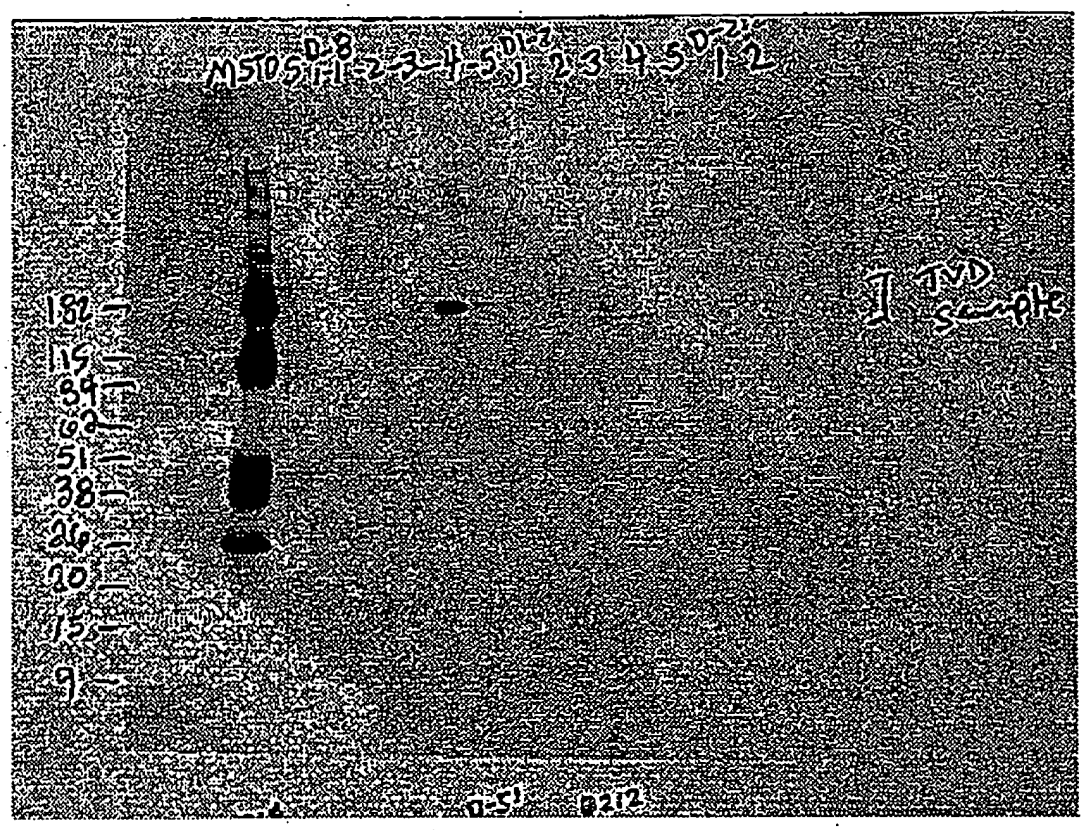


Western blot of TVD (Shybrid) plant tissue showing M=molecular weight markers, STD=virus standard, E=non-transformed plant tissue, the numbers represent individual plants. Same blot as before - closer view.



Western blot of TVD (Shybrid) plant tissue showing M=molecular weight markers, STD=virus standard, E=non-transformed plant tissue, the numbers represent individual plants. This blot show the higher expressing plant.

This blot and all subsequent blot have identical amounts of viral standard loaded (5 ul of a commercially available TGEV vaccine from AMBICO)



Western blot of TVD (Shybrid) plant tissue showing M=molecular weight markers, STD=virus standard, E=non-transformed plant tissue, the numbers represent individual plants. Further back picture as previous picture.